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Transcriptional Regulation of Two Serum-Induced RNAs in Mouse Fibroblasts: Equivalence of One Species to B2 Repetitive Elements

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We obtained eight cDNA clones that define five genes whose expression (appearance of transcripts in the cytoplasm) is enhanced when quiescent mouse fibroblasts are stimulated with serum to divide. Two of these clones (designated 49C8 and 16C8) correspond to RNA species that are present in the cytoplasm of quiescent cells at very low levels. After serum stimulation, the level of 16C8 mRNA rose more rapidly than that of 49C8 RNA, reaching a maximum around 6 to 12 h. The data suggest that 49C8 and 16C8 RNAs are induced as a result of independent stimuli. Either fibroblast growth factor or 12-tetradecanoylphorbol-13-acetate alone could induce 16C8 expression almost as effectively as serum; in contrast, 49C8 was not efficiently induced by epidermal growth factor, fibroblast growth factor, insulin, or 12-tetradecanoylphorbol-13-acetate. Inhibitors of transcription and translation diminished the induction of 16C8, while 49C8 expression was sensitive to actinomycin D but not cycloheximide or 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. In vitro transcription experiments with isolated nuclei revealed a peak in transcriptional activity of the 16C8 gene at around 3 h after serum stimulation. Sequence analysis of the 49C8 cDNA clone showed >90% homology of a large portion to a consensus rodent B2 repetitive element.

The expression of particular genes during the G_1 phase of the mammalian cell cycle is required for the commitment of the cell towards division (3, 4, 40). Genes that possess the characteristic of enhanced expression when quiescent cells in culture are stimulated to proliferate by mitogens therefore constitute an interesting class in which functions that are essential for cell proliferation may be sought. Information about changes in gene expression during G_1 is currently being derived from two approaches. Firstly, the availability of cloned proto-oncogenes and a cellular tumor antigen (p53) has enabled many laboratories to study the activation of these important genes in mitogen-stimulated cells (10, 16, 20, 27, 32). There is a temporal order of induction of these genes, with c-*fos* activation being rapid and transient and preceding the activation of c-*myc* (27), which in turn precedes the activation of p53 (32).

A second approach has involved the use of cDNA libraries to look for genes that are preferentially expressed in G_1 (6, 9, 12, 22). Cochran et al. (6) reported an initial characterization of some 46 platelet-derived growth factor-inducible clones that represent five independent gene sequences. Two clones (KC and JE) correspond to RNAs whose accumulation was (i) sensitive to an inhibitor of transcription (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; DRB) and (ii) enhanced ("superinduced") by an inhibitor of translation (cycloheximide [CHM]). A third clone (JB) had partial homology with the *fos* oncogene (7). Using serum to stimulate quiescent BALB/c-3T3 cells, Linzer and Nathans (22) detected several cDNA clones corresponding to induced mRNAs, one of which, called proliferin, was found to be a member of the prolactin growth hormone family (23).

We screened about 8,000 clones in a cDNA library derived from a poly(A)$^+$ mRNA preparation from secondary cultures of mouse embryo fibroblasts to isolate cDNA clones whose cognate RNAs are present in elevated levels in cytoplasmic RNA from serum-stimulated cells. The present work describes some of these serum-induced cDNA clones. One group of clones corresponding to serum-induced RNAs was found to have homology to the murine B2 repetitive element (1). Recently, these sequences have been found associated with a class I major histocompatibility antigen gene that is induced in transformed mouse cells (37).

MATERIALS AND METHODS

Cell culture. Subconfluent cultures (2×10^6 cells per 15-cm dish) of Swiss 3T3 fibroblasts (American Type Culture Collection) were rendered quiescent by incubation for 4 days in Dulbecco modified Eagle medium containing 0.5% fetal bovine serum obtained from GIBCO Laboratories. Medium was changed after 2 days. Cells were stimulated in medium containing 10% fetal bovine serum. DNA synthesis was monitored in quiescent cells and at 18 h after serum stimulation as described previously (9).

RNA isolation, electrophoresis, blotting, and hybridization. RNA was isolated as previously described (9), and equivalent amounts were electrophoresed through 1.1% agarose gels in a morpholinopropanesulfonic acid (MOPS)-acetate buffer containing 2.2 M formaldehyde (24) for 3 to 4 h at 50 V (70 mA). RNA was transferred to nitrocellulose, and hybridization and autoradiography were as described previously (9). Nick-translated plasmid DNA probes were used at 10^8 cpm/μg. When blots were used for more than one hybridization, bound probe was removed by incubation in hybridization solution at 75°C for 5 min. Densitometry was performed as previously described (9).

Preparation of nuclear RNA and slot-blotting transfer. The nuclear pellet obtained from the second 800 × *g* centrifugation step in the procedure that we have previously used for the purification of cytoplasmic RNA (9) was used as the source of nuclear RNA. The guanidinium isothiocyanate-hot phenol method was employed (24). After ethanol precipitation, the resulting nucleic acid pellet was suspended in sterile

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water, and A_{260} was monitored. Samples were adjusted to 20 mM Tris hydrochloride (pH 7.6)–4 mM $MgCl_2$–20 mM NaCl–10 mM vanadyl ribonucleosides (1) and digested with 50 ng of DNase I (ribonuclease free; Worthington Diagnostics) per μg of nucleic acid for 30 min on ice. Equivalent amounts (as determined from the initial A_{260} measurement) were transferred to nitrocellulose with a Schleicher & Schuell Minifold II slot-blot apparatus. The RNA was denatured in 6.15 M formaldehyde–10× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) for 15 min at 65°C in a volume of 400 μl before transferring to nitrocellulose that had been preequilibrated in 10× SSC. Filters were treated for hybridization as described previously (9).

In vitro runoff transcription, RNA isolation, and hybridization. Nuclear runoff transcription reactions were performed with isolated nuclei and [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear Corp.) as described by Greenberg and Ziff (10) except that 2×10^7 nuclei were used in each 200-μl reaction. After 15 min at 30°C, the ^{32}P-labeled RNA product was isolated by guanidinium isothiocyanate-hot phenol extraction (24), followed by trichloroacetic acid precipitation (11). Hybridizations were performed with 1×10^6 to 3×10^6 cpm per filter on replica filters prepared by slot-blotting 1-μg samples of plasmid DNA purified by ethidium bromide-cesium chloride isopycnic centrifugation. The DNA samples were transferred to nitrocellulose by the method of Kafatos et al. (15) but without prior conversion to linear molecules. The sheets were hybridized at 42°C in 50% formamide for 48 h and then washed as described for Northern blots.

RESULTS

Serum inducibility of specific mRNAs. Edwards and Denhardt (9) have reported previously on the preparation and screening of a cDNA library that had been derived from poly(A)$^+$ mRNA from growing subconfluent cultures of secondary mouse embryo fibroblasts and enriched for rare sequences. Differential colony hybridization uncovered eight clones, out of approximately 8,000 screened, that correspond to RNAs whose abundance is reproducibly greater in the cytoplasm of serum-stimulated cells, compared with quiescent cells. These clones identify five distinct genes (Table 1). In the work described here, we used low-passage-number cultures of Swiss 3T3 fibroblasts in our synchrony experiments, since, with one exception, the levels of mRNAs corresponding to our various cDNA clones are equivalent in mouse embryo fibroblasts and Swiss 3T3 fibroblasts (data not shown) and since these cells afford superior synchronization, i.e., fewer cycling cells in the quiescent population and a greater percentage of cells responding to the serum stimulus.

TABLE 1. Serum-induced cDNA clones

Clone[a]	mRNA size (bases)	Gene product size (kilodaltons)	Time of maximal induction (h)[b]
[5B10 99H10]	680	7 (15)[c]	S
16C8	900	18	6–12
[49C8 29H9 7E4]	550		S
31H4	2,100	48?	6–18
33H1	2,000	42	6–12

[a] Brackets enclose clones that were found to cross-hybridize.
[b] S, The signal reached its highest value during S phase (i.e., 12 to 24 h after serum stimulation). No subsequent decline was visible, perhaps due to loss of synchrony.
[c] A small amount of a second polypeptide was seen at this position in the SDS gel.

Figure 1 shows a Northern blot analysis of total cytoplasmic RNA isolated from Swiss 3T3 fibroblasts at various times after serum stimulation. The hybridization signals from a variety of nick-translated cDNA clones are shown. Panels a and c show the accumulation of two serum-induced RNA species with approximate sizes of 0.9 and 0.55 kilobases corresponding to clones 16C8 and 49C8, respectively. Panel b contrasts two clones that have previously been shown to increase (5B10) and decrease (69H6) in RNA preparations from serum-stimulated mouse embryo fibroblasts (9). 5B10 mRNA was detectable in RNA from quiescent cells (in longer exposures than that shown here), and its level showed a three- to fourfold enhancement after serum stimulation (9). RNA corresponding to 16C8 and 49C8 was present in cytoplasmic RNA from quiescent 3T3 cells in trace or undetectable amounts, with variability presumably due to the degree of quiescence of the cell population.

Two control clones are shown in Fig. 1d. Clone 5D12 corresponds to an ~0.5-kilobase RNA species whose total cytoplasmic concentration does not change appreciably; the slight increase in 5D12 RNA abundance largely reflects the increase in the poly(A)$^+$ mRNA-to-rRNA ratio in cytoplasmic RNA after serum stimulation (14), since the relative abundance of 5D12 RNA does not vary within the poly(A)$^+$ mRNA class (9). Actin mRNA, identified by its hybridization with the β-actin clone pA1 (5), is detectable in quiescent cells but shows an increase in concentration in G_1, as has been described previously (33). Table 1 summarizes data on our clones regarding the sizes of their corresponding mRNAs, their translation products (as detected by hybrid selection and in vitro translation), and their times of maximal induction. The set of clones of which 49C8 is the prototype did not hybrid select translatable RNAs.

Association of serum-induced RNAs with polysomes. To establish whether the 49C8 RNA was in fact translated within the cell, we looked for its presence in polyribosomes. To this end, the cytoplasmic constituents of cells at 0, 6, and 18 h after serum stimulation were sedimented on linear sucrose gradients; the resulting polysome profiles are shown in the upper panels of Fig. 2. Fractions from these gradients were pooled in seven groups (A through G) as indicated. The RNA was extracted, and a Northern blot was prepared to which nick-translated probes from a cDNA clone encoding mouse glyceraldehyde-3-phosphate dehydrogenase (MGAP) and from clones 16C8, 49C8, and 69H6 were hybridized (Fig. 2, lower panels).

MGAP was included as a control since the relative level of MGAP poly(A)$^+$ mRNA does not change greatly after serum stimulation (9), and the size of its mRNA (1.4 kilobase) distinguishes it from 16C8 and 49C8. RNA corresponding to MGAP was found on polysomes (fractions D through G in each gradient) at all times. In contrast, 16C8 mRNA was only apparent after growth stimulation and was associated with polysomes at both 6 and 18 h. (Note that equivalent amounts of material were not applied to each gradient, and therefore, quantitative comparisons between gradients should not be made.) Figure 2 also shows that 49C8 RNA was found predominantly in the submonosomal region of the gradient (fractions A and B) at 18 h, at which time its cytoplasmic concentration was maximal (Fig. 1). A very small amount of material was present in fractions C and D.

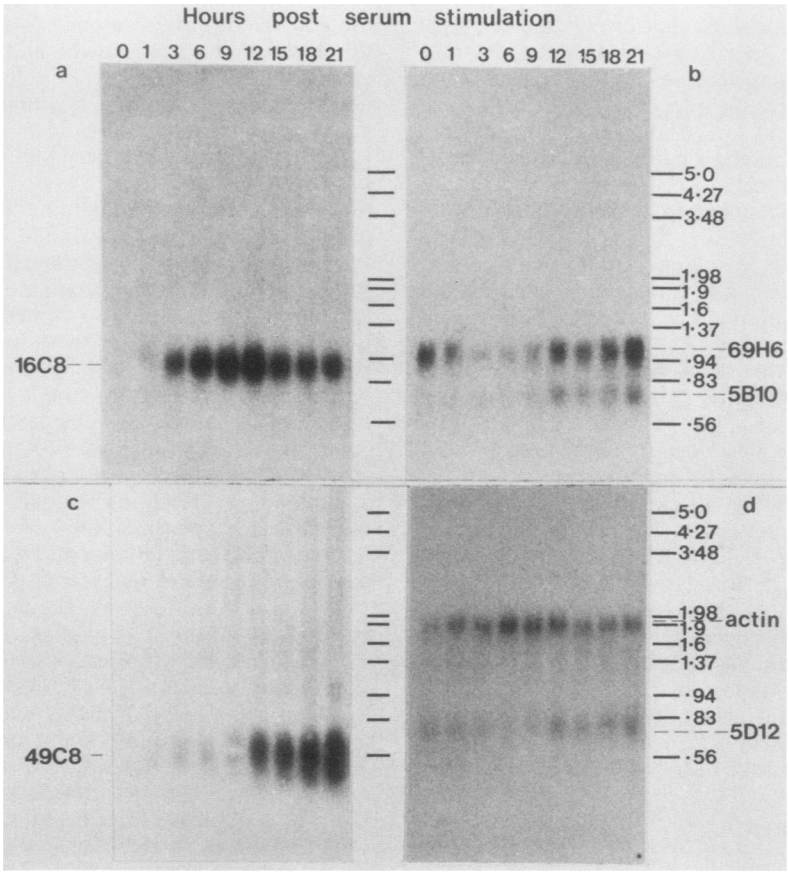

FIG. 1. Induction of specific RNAs in serum-stimulated Swiss 3T3 cells. Total cytoplasmic RNA was isolated from Swiss 3T3 fibroblasts at the indicated times after stimulation of quiescent cells with medium containing 10% fetal bovine serum. Equal amounts (10 μg per lane) were electrophoresed on agarose gels and transferred to nitrocellulose as described in Materials and Methods. Blots were hybridized with the following nick-translated probes: 16C8 (a), 69H6 and 5B10 (b), 49C8 (c), and 5D12 and pA1 (d). The pA1 plasmid contains chicken β-actin cDNA (5). Size standards used in all Northern blots were a product of an *Hin*dIII and *Eco*RI double digest of λ DNA.

From these data, we inferred that 49C8 RNA was not found in association with polysomes and, therefore, probably does not encode a protein. RNA corresponding to 69H6 (shown as an additional panel at the bottom of Fig. 2) was found mostly in fraction B, with minor amounts sedimenting in the polysome region, indicating that 69H6 RNA, which is fairly abundant with the poly(A)$^+$ mRNA class (~0.25%), perhaps exists predominantly as untranslated ribonucleoprotein.

Effects of other mitogens on the levels of 16C8 and 49C8 expression. For effective simultaneous comparison of both 49C8 and 16C8 RNA levels in mitogen-stimulated Swiss 3T3 cells, we chose to look at RNA isolated from cells at 12 h after stimulation, since at this time both 16C8 and 49C8 levels are close to their maxima (Fig. 1). DNA synthesis under these conditions begins at about 12 h and peaks at 20 h. The gel from which the blot in Fig. 3 was obtained contained equal amounts of total cytoplasmic RNA from resting cells (lane a), cells 12 h after stimulation with 10% serum (lane b), and cells 12 h after stimulation with 0.5% serum supplemented with epidermal growth factor (EGF; lane c), fibroblast growth factor (FGF; lane d), insulin (lane e), or 12-*O*-tetradecanoylphorbol-13-acetate (TPA; lane f). Figure 3 shows the hybridization signals obtained with MGAP, 16C8, and 49C8 probes.

We performed densitometry on the autoradiograph shown in Fig. 3. After subtracting the signal generated by RNA from quiescent cells (in this experiment there was detectable 49C8 RNA in the unstimulated cells), we estimated the degree of induction of 49C8 and 16C8 RNAs as a percentage of that obtained after stimulation of cells with medium containing 10% serum. For 16C8, these values were 15.5, 51.2, 11.9, and 78.5% for EGF, FGF, insulin, and TPA, respectively. For 49C8, the corresponding values were 10.3, 2.7, 0, and 1.4%. None of the individual mitogens was as effective as 10% serum in eliciting the full induction of 49C8 RNA, whereas both TPA and FGF gave a substantial induction of 16C8 RNA. These results imply that the induction pathways of 49C8 and 16C8 are different and that induction of 49C8 RNA may require the presence of several mitogens.

Effects of inhibitors of transcription and translation. Changes in gene expression can be effected at various levels that can be broadly classified into transcriptional, posttranscriptional, translational, and posttranslational. One approach to identifying the level of control is through the use of inhibitors. For example, the regulation of RNAs whose accumulation is sensitive to inhibitors of RNA polymerase II is assumed to occur at the transcriptional level. Inhibitors of protein synthesis in some cases cause an enhanced accumulation of the RNA under study (known as superinduction), perhaps because the translation of the molecule is inhibited and the RNA is thereby protected or because the synthesis of a labile repressor protein is blocked. To determine whether the RNAs of interest here are subject to this

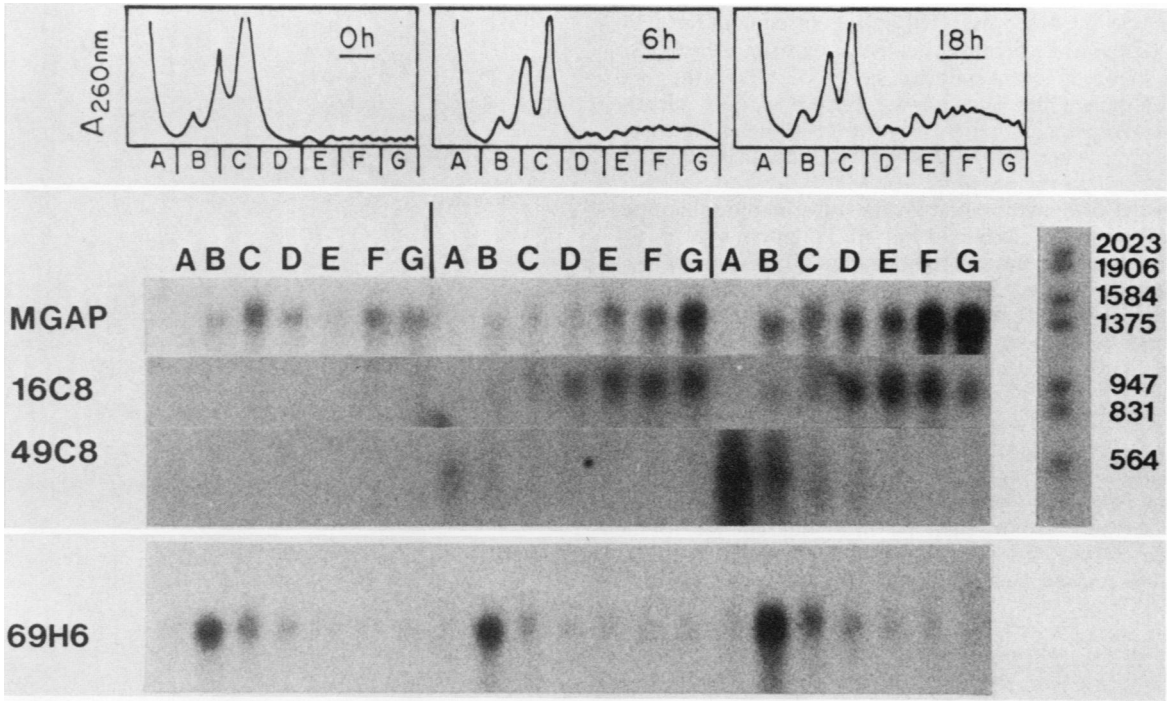

FIG. 2. Cellular distribution of 16C8 and 49C8 RNAs. The cytoplasmic fractions from cells at 0, 6, and 18 h after serum stimulation were fractionated by velocity sedimentation on linear sucrose gradients as described in Materials and Methods. A_{260} was monitored, and the resulting profiles, sedimentation towards the right, are shown in the top panels of the figure. Regions of the gradients were pooled into seven fractions (A through G) which were analyzed on Northern blots. The autoradiographs show the results of hybridization ot 49C8, 16C8, and MGAP and 69H6 nick-translated probes. The same blot was hybridized sequentially with the four probes. The blots for MGAP, 16C8, and 49C8 but not 69H6 are placed so that the approximate sizes can be determined from the denatured λ DNA markers on the right.

regulation, we assessed the effect of a 6-h treatment with CHM, actinomycin D, or DRB upon the induction of 16C8 and 49C8 RNAs in serum-stimulated cells.

Figure 4 shows a Northern blot analysis with the same probes used in Fig. 2 and 3. It is clear that the 49C8 and 16C8 RNA levels in the cytoplasm were differentially affected by the inhibitor treatment. Induction of 16C8 was striking by 6 h after serum stimulation (lane b) but substantially inhibited by CHM, DRB, and actinomycin D (lanes d, e, and f, respectively). Relative to the amount of 16C8 mRNA in lane b, we estimate that the extent of inhibition was 84% for CHM and essentially complete for DRB and actinomycin D. In other experiments, we have shown that α-amanitin (1 μg/ml) inhibited the induction of 16C8 mRNA (data not shown). In contrast, 49C8, whose cytoplasmic concentration was not maximal at the 6-h time point (Fig. 1), showed an increased accumulation in the presence of both CHM (twofold) and DRB (fourfold). A 6-h treatment of unstimulated cells with CHM (without changing the medium) also led to induction of 49C8 RNA to 81% of the amount observed after serum stimulation. Since actinomycin D abolished the induction of 49C8 RNA by serum (lane f) but DRB (lane e) did not, we deduce that transcription that is not performed by RNA polymerase II is required. The control clone MGAP showed only minor fluctuations through this series of treatments.

Runoff transcription studies in isolated nuclei. One interpretation of the data presented in Fig. 4 is that 16C8 gene expression is controlled at the transcriptional level. If so, it should be possible to observe increased transcriptional activity of the 16C8 gene after serum stimulation. To quantify the transcription of these genes, we performed nuclear runoff transcription reactions with nuclei isolated from cells at various times before and after serum stimulation. This technique has found widespread application recently (10, 11), since the RNA that is synthesized accurately reflects the transcriptional state of the cell (8).

We used the runoff RNA transcripts to probe identical blots carrying plasmid DNAs from selected clones (Fig. 5). Included in this set are clones corresponding to MGAP, actin, tubulin, and a portion of mouse 18S rRNA (D. R. Edwards, unpublished observations). The strongest signals were obtained from the 18S rRNA clone, although 49C8 also gave very stong signals. Transcription of sequences homologous to the 49C8 clone was largely unaffected by the growth state of the cell. Transcription of the 16C8 gene was low in quiescent cells although detectable above the signal generated by pBR322. A longer exposure is shown at the bottom of the figure. In contrast to the relatively constant transcription of MGAP and tubulin, a transient increase in the rate of transcription of 16C8 was apparent at 3 h post-serum stimulation. This brief transcriptional burst may be responsible for the increased cytoplasmic abundance of 16C8 mRNA at 6 to 12 h after serum stimulation.

Presence of serum-induced transcripts in nuclear RNA. To complement the transcriptional studies shown in Fig. 5, we analyzed the relative steady-state abundance of transcripts homologous to control and serum-induced cDNA clones in nuclear RNA from quiescent and serum-stimulated cells. Equivalent amounts of nuclear RNA that had been pretreated with DNase I as described in Materials and Methods were applied to nitrocellulose, and the blots were hybridized with nick-translated plasmid DNA probes (Fig. 6A). For most of the DNAs tested, the results reflected constant levels of their transcripts in nuclei at all growth stages. An

exception was provided by MRP (mitogen-regulated protein [29]). This clone has recently been shown to correspond to proliferin (23), a serum-induced mRNA in BALB/c and Swiss 3T3 cells (30). The gene(s) encoding MRP clearly displayed evidence of enhanced transcription after serum stimulation. This has also been shown in runoff transcription experiments (data not shown).

The results of densitometry on the autoradiographic data shown in Fig. 6A are shown in Fig. 6B. Figure 6 shows that 16C8 transcripts did not change significantly in their abundance in nuclei after serum stimulation, as also shown by the control clone, MGAP. At first blush, these data are seemingly at odds with the inhibitory effect of DRB upon induction of 16C8 mRNA after serum stimulation (Fig. 4) and the transcriptional activation seen in the nuclear runoff experiments of Fig. 5. The results could be reconciled, however, if there were a low-level constitutive transcription of the 16C8 gene that was enhanced by serum, accompanied at the same time by more efficient processing and transport of the new transcripts; there would then be little net change in the abundance of 16C8 RNA in the nucleus.

Equivalence of 49C8 cDNA clones with mouse B2 repetitive elements. Analysis of the 49C8 sequence revealed that it contained a 195-base-pair insert whose sequence had extensive homology with an abundant dispersed repetitive element designated B2 (19). The two sequences are shown in Fig. 7. The 49C8 sequence commences at nucleotide 38 of the B2 consensus, indicating that this clone is incomplete. Two groups have obtained evidence that the short, dispersed-size class of cytoplasmic B2 transcripts are the products of RNA polymerase III (18, 38). Singh et al. (38) also showed that these transcripts possess similar 5′ termini but differ at their 3′ ends, presumably due to the use of different termination signals or degradation. Our other B2 clones (29H9 and 7E4; Table 1) have been shown to be imperfect matches of the 49C8 sequence by cross-hybridization experiments, followed by washing at high stringency (data not shown).

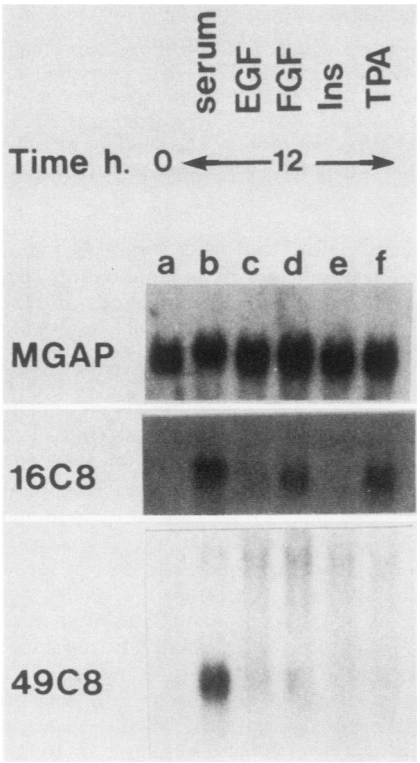

FIG. 3. Induction of 16C8 and 49C8 RNAs by individual mitogens. Total cytoplasmic RNA was prepared from quiescent Swiss 3T3 cells (lane a) and cells after 12 h in the presence of various mitogenic stimuli (lanes b through f). A Northern transfer was prepared as described earlier, with 10 μg of RNA per lane. The blot was hybridized sequentially with nick-translated probes from clones 49C8, 16C8, and (lastly) MGAP. Between successive hybridizations, blots were washed, and bound probe was eluted as described in Materials and Methods. Lanes contained Dulbecco modified Eagle medium plus: b, 10% fetal bovine serum; c through f, 0.5% serum supplemented with 100 ng of EGF per ml; 50 ng of FGF per ml; 3 μg of insulin (Ins) per ml; and 100 ng of TPA per ml, respectively.

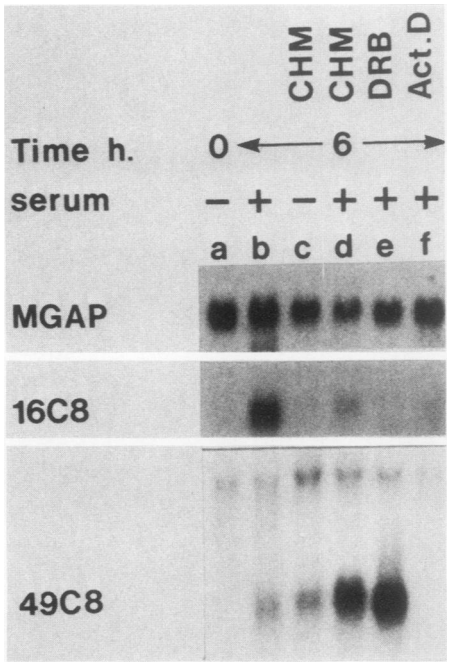

FIG. 4. Effects of inhibitors of transcription and translation upon the induction of 49C8 and 16C8 RNAs. A Northern blot analysis was carried out with cytoplasmic RNA as described in the legend to Fig. 3. The following treatments are shown: lane a, quiescent cells in medium containing 0.5% serum; lane b, cells stimulated for 6 h in medium containing 10% serum; lane c, cells treated for 6 h in medium plus 0.5% serum and 10 μg of CHM per ml; lane d, as for lane b plus 10 μg of CHM per ml; lane e, as for lane b plus 25 μg of DRB per ml; lane f, as for lane b plus 2 μg of actinomycin D (Act. D) per ml.

DISCUSSION

The changes in gene expression that take place when quiescent mammalian cells are stimulated to proliferate have begun to be analyzed at the level of individual genes. In this publication, we describe cDNA clones that we have identified as corresponding to RNA species that are induced after serum stimulation of quiescent mouse fibroblasts. Two other laboratories have also generated collections of mouse cDNA clones whose cognate RNAs are growth factor-induced either by platelet-derived growth factor alone (6) or by serum (22). Similarly, Hirschhorn et al. (12) identified five cDNA clones from a temperature-sensitive cell line derived from baby hamster kidney cells which corresponded to mRNAs

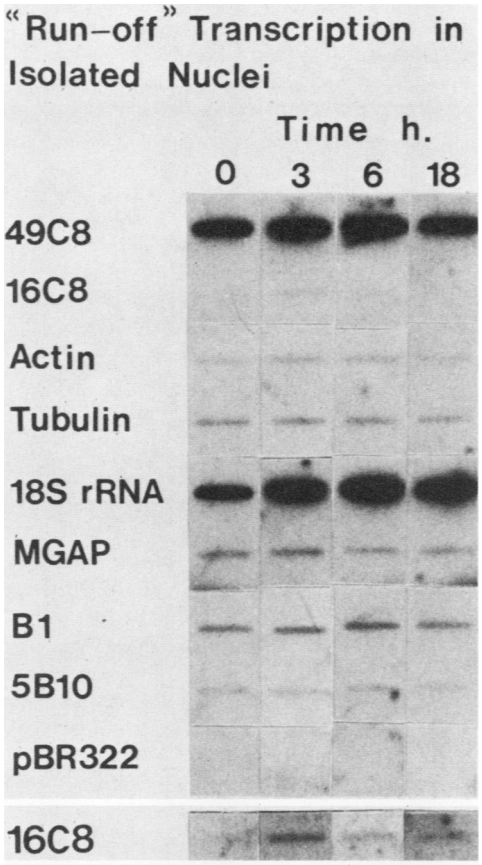

FIG. 5. Transcriptional analysis of 49C8 and 16C8 genes in isolated nuclei. Nuclei from approximately 2×10^7 cells were prepared from Swiss 3T3 cells at 0, 3, 6, and 18 h after serum stimulation. Filters carrying the indicated plasmid DNAs were hybridized to ^{32}P-labeled nuclear runoff transcript probes (3×10^6 cpm per filter) that had been prepared as described in Materials and Methods. The sources of the clones were as follows: actin (p749), a rat skeletal muscle actin, D. Yaffe; chicken α-tubulin (pT1), D. Cleveland (5); MGAP, P. Curtis; a murine B1 repeat, N. Hastie.

whose abundance was augmented three- to sixfold after stimulation of quiescent cells with serum. Transcripts homologous to repetitive VL30 elements are induced when quiescent AKR mouse cells are stimulated with serum or EGF and insulin (13).

All these clones, including ours, were detected with labeled cDNA by a differential colony hybridization technique only able to detect sequences more abundant than about 0.1% of the mRNA population; low-abundance sequences (less than 100 copies per cell) were not detected. It seems likely, therefore, that among the two to three dozen clones so far described, several will turn out to represent the same mRNA. Definitive identification awaits DNA sequence information on these clones, which so far is available only for proliferin, a 1-kilobase mRNA induced by serum (23), and JB, which is related to the *fos* oncogene (7).

For our cDNA clones, we have monitored such characteristics as the sizes of their corresponding RNAs and translation products (Table 1), their inducibility by individual mitogens (Fig. 3), and the effects of inhibitors of transcription and translation upon their induction (Fig. 4). Clone 16C8 corresponds to a ~900-nucleotide-long mRNA that translates in vitro to yield a protein of M_r 18,000. DNA sequencing analysis (data not shown) shows that this clone does not share identity with proliferin (22, 23) and is not related to any protein in the NIH protein sequence library (September 1984). On the basis of these and other characteristics (nonsuperinducibility by CHM, induction by TPA and FGF), we conclude that 16C8 represents a novel mitogen-induced gene.

Appearance of 16C8 mRNA in the cytoplasm after serum stimulation is substantially (~90%) inhibited by DRB (Fig. 4), and a peak of 16C8 gene transcription was observed 3 h after serum stimulation (Fig. 5). These data argue for transcriptional regulation of the 16C8 gene. Analysis of nuclear RNA revealed similar concentrations of 16C8 transcripts in all growth states (Fig. 6). One possible interpretation of this finding is offered by the work of Darnell and co-workers, who have studied the synthesis and turnover of nuclear RNA in Chinese hamster ovary cells (35, 36). Salditt-Georgieff et al. (36) showed that heterogeneous nuclear RNA contained three times as many 5′-methylated caps as 3′-poly(A)$^+$ tails and that most (two-thirds) of these 5′-capped molecules did not subsequently enter polysomes. Thus, most primary nuclear transcripts in mammalian cells are not destined to become cytoplasmic mRNA molecules. We are investigating the distribution of 16C8 sequences between nuclear poly(A)$^+$ and poly(A)$^-$ RNAs.

The set of clones identified by the prototype 49C8 corresponds to small cytoplasmic RNAs of the B2 class (19). B2 elements are transcribed by both RNA polymerases II and III (18, 38). Most of the cytoplasmic RNA species that hybridize to B2 DNA probes such as 49C8 are of the short (~500 nucleotide)-size class (34), and evidence has been presented that these species are products of RNA polymerase III (18, 38). In harmony with this view, we showed (Fig. 4) that the serum induction of the B2 RNAs was resistant to DRB but sensitive to actinomycin D.

Scott et al. (37) have discovered a B2 sequence in the 3′ noncoding region of a class I major histocompatibility antigen that is induced in cells transformed by a variety of viral or chemical agents. Murphy et al. (28) have analyzed total fetal RNA at different stages of mouse embryogenesis and shown that transcripts homologous to B2 peak sharply at the 9- to 10-day stage. These authors also observed that in cytoplasmic poly(A)$^+$ mRNA from undifferentiated embryonal carcinoma cells, a B2 probe detected a broad size range of mRNAs whose abundance decreased upon induction of differentiation. These ideas have led to speculation that the B2 elements may identify mRNAs that are activated in oncogenesis and embryogenesis (2, 28). The role of the short cytoplasmic transcripts generated by RNA polymerase III is uncertain, although it is clear that they are also induced after simian virus 40 transformation of mouse cells (37, 38). Our data show that these sequences are induced by serum in secondary cultures of mouse embryo fibroblasts (9) and also in established Swiss 3T3 fibroblasts. At present, it is not known whether expression of the short cytoplasmic B2 transcripts is functionally required for cell proliferation or transformation. This will clearly be the goal of future studies upon these RNAs.

We can separate the induction of 49C8 (B2) and 16C8 RNAs by their response to different mitogens (Fig. 3). Therefore, these genes can be used as markers for the activation of independent processes by specific stimuli. It has been appreciated for some time that in murine fibroblasts growth factors activate specific processes in a sequence of steps as cells emerge from quiescence (31). Platelet-derived growth factor and FGF are ascribed competence-promoting activity (39), while EGF and somatomedin C affect transition

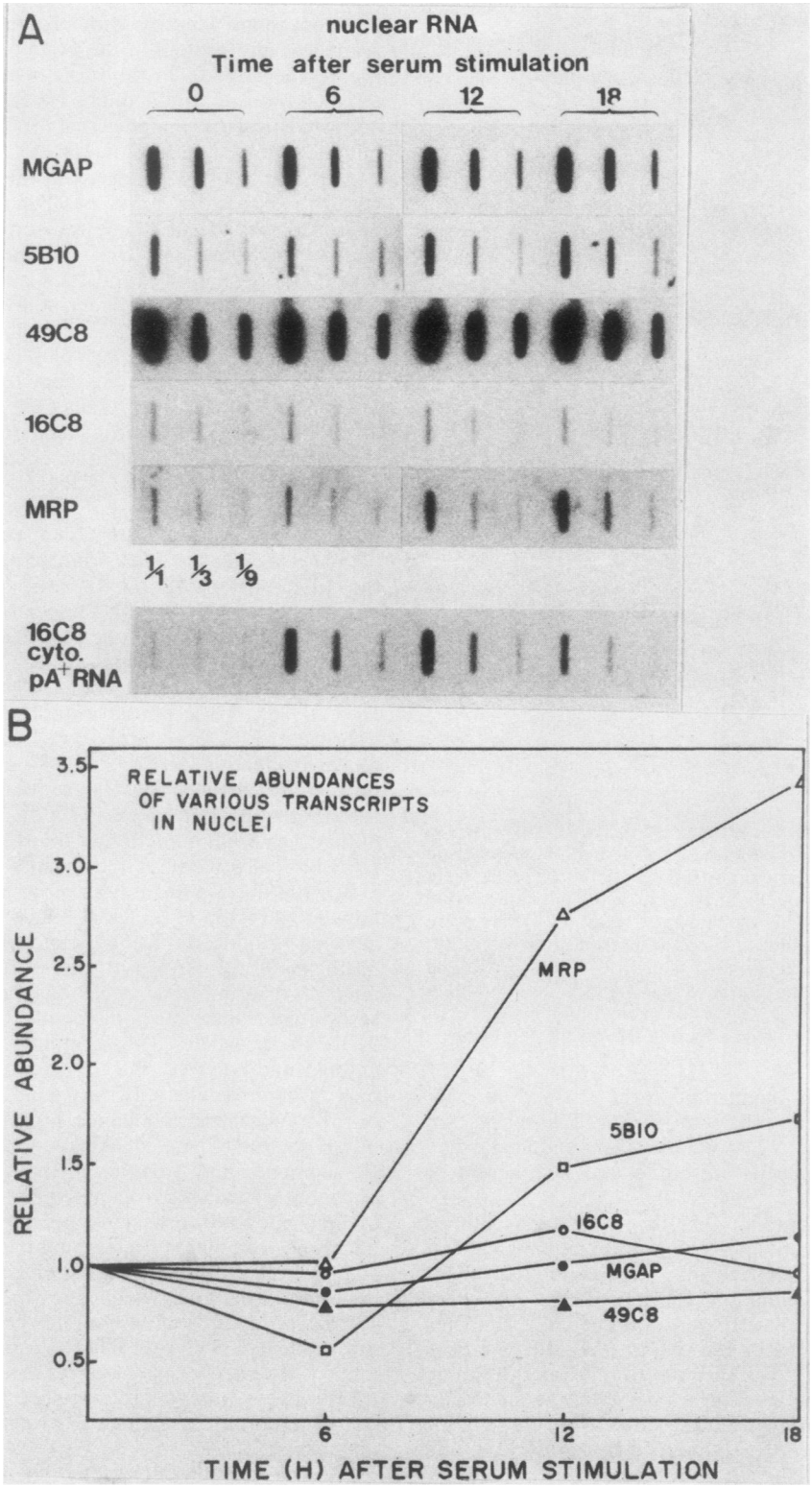

FIG. 6. Relative levels of transcripts homologous to serum-induced clones in nuclei. Nuclear RNA was prepared from quiescent Swiss 3T3 cells and cells at 6, 12, and 18 h after serum stimulation as described in Materials and Methods. For each RNA preparation, three samples were applied to nitrocellulose in a threefold dilution series, with the maximum amount of RNA applied being 3 μg. Control experiments in which the RNA samples were hydrolyzed in 0.25 M NaOH at 65°C for 30 min confirmed that in each case the hybridization signals were not a result of contaminating DNA (data not shown). Identical blots were hybridized to the indicated nick-translated probes (A). Exposure times varied from 6 h (for 49C8) to 4 days (for 16C8). A control blot carrying cytoplasmic poly(A)$^+$ mRNA isolated from cells at 0, 6, 12, and 18 h after serum stimulation was probed with 16C8 (16C8 cyto. pA$^+$RNA). The autoradiographic signals were quantified by densitometry, and the results are shown in panel B.

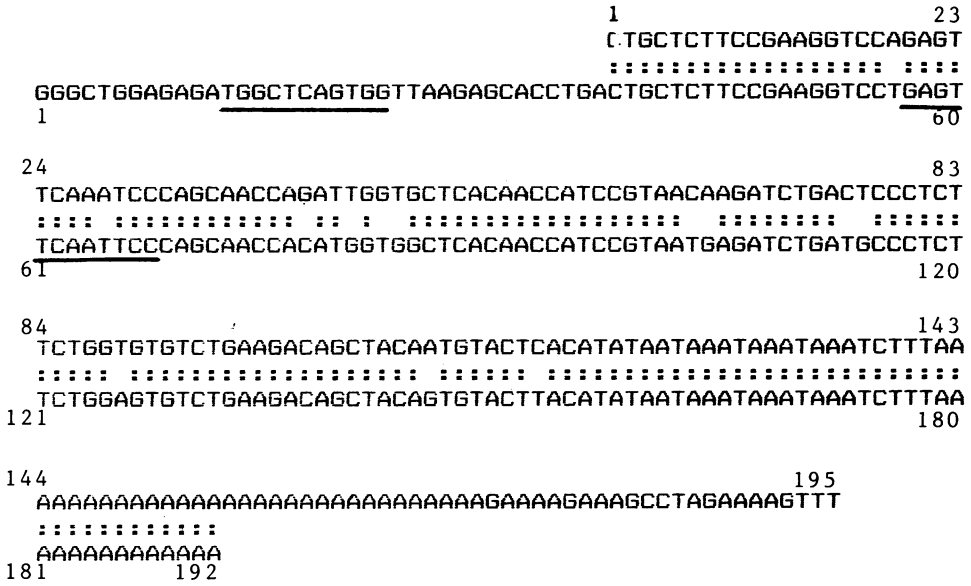

FIG. 7. Sequence comparison of 49C8 with the consensus rodent B2 element. The top line shows the sequence obtained from 49C8 by dideoxy sequencing after subcloning into M13mp8. The lower line is the consensus rodent B2 sequence (19). The consensus sequences of the A- and B-boxes of the RNA polymerase III split promoter (19) are underlined.

beyond competence to a second control point "V" (21). Somatomedin C alone or a hyperphysiological concentration of insulin is required for progression past V (21). We are analyzing the induction of 16C8 and 49C8 (B2) with an extended range of mitogens, in particular to determine whether 16C8 is a marker for competence, as its induction by FGF may imply, and whether 49C8 (B2) is related to some later progression event.

There can no longer be any doubt that certain genes are expressed at specific times during G_1. Studies of oncogenes have revealed a temporal order of gene activation (10, 16, 27, 32), and the products of certain genes (p53 [25] and c-*ras* [26]) have been shown to be necessary for cell cycle progression. Protein synthesis is required for the full induction of 16C8 expression (Fig. 4). Thus, we suggest that 16C8 expression is not a primary consequence of the stimulation of quiescent cells by competence-promoting growth factors (e.g., platelet-derived growth factor and FGF). It is possible that the products of early gene expression may function as transcriptional activators of later gene expression. In this respect, it is interesting that the product of a rearranged c-*myc* gene has been shown to be capable of stimulating the expression of a mammalian heat shock protein (17).

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